Journal of Cellular Biochemistry 50:124–129 (1992)

ARTICLES

The Rapid Nongenomic Actions of 1α,25-Dihydroxyvitamin D_3 Modulate the Hormone-Induced Increments in Osteocalcin Gene Transcription in Osteoblast-Like Cells

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Abstract We have previously shown that one of the rapid nongenomic actions of 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), the increase in intracellular calcium (Ca^{2+}), accompanies the increased osteocalcin (OC) mRNA steady-state levels in rat osteosarcoma cells. To determine the functional significance of the nongenomic actions, we have measured changes in intracellular Ca^{2+} as an indicator of the rapid effects and have assessed the effect of inhibition of the rapid increase in cellular Ca^{2+} by the inactive epimer, 1β,25-dihydroxyvitamin D_3 (1β,25-$(OH)_2D_3$), on OC mRNA steady-state levels and transcription. 1β,25-dihydroxyvitamin D_3 inhibited 1α,25-$(OH)_2D_3$ induced increases in intracellular Ca^{2+} and OC mRNA transcription at 1 hr and OC mRNA steady state levels at 3 hr. 1β,25-Dihydroxyvitamin D_3 did not alter the binding of the vitamin D receptor complex to the vitamin D responsive element of the OC gene. The results demonstrate the functional importance of the rapid, nongenomic actions of 1α,25-$(OH)_2D_3$ in the genomic activation of the OC gene by the hormone in rat osteoblast-like cells, perhaps by modifying subtle structural and/or functional properties of the vitamin D–receptor DNA complex or by affecting other protein DNA interactions that support OC gene transcription.

Key words: intracellular Ca^{2+}, 1β,25-$(OH)_2D_3$, ROS 17/2.8, OC mRNA

1α,25-Dihydroxyvitamin D_3 rapidly increases intracellular Ca^{2+} in mouse osteoblasts [Lieberherr, 1987] and rat osteoblast-like cells [Civitelli et al., 1990; Baran et al., 1991]. This rapid effect of the hormone on intracellular Ca^{2+} does not require the classic vitamin D receptor based on observations that (1) 1α,25-$(OH)_2D_3$ increases cell Ca^{2+} in osteoblast-like cells (ROS 24/1) that lack the receptor, and (2) the effect is inhibited by the inactive epimer, 1β,25-$(OH)_2D_3$, which does not displace 1α,25-$(OH)_2D_3$ from the classic receptor [Baran et al., 1991]. Although it has been suggested that modulation of intracellular Ca^{2+} might represent a mechanism whereby the cell integrates different input signals, so as to be selectively conditioned to respond to subsequent stimulations [Civitelli et al., 1990], a functional role for the rapid nongenomic effects of 1α,25-$(OH)_2D_3$ in osteoblast-like cells has not been clearly established. Such a role has been suggested by the observation that 1α,25-$(OH)_2D_3$ induced up-regulation of its receptor in UMR106 cells is inhibited by Ca^{2+} channel blockers, chelation of extracellular Ca^{2+}, and inhibition of intracellular Ca^{2+} release [Van Leeuwen et al., 1990].

The present study demonstrates that 1β,25-$(OH)_2D_3$, which has no effect on basal OC transcription, does inhibit the 1α,25-$(OH)_2D_3$-induced rise in intracellular Ca^{2+} and blocks the hormone-induced increases in OC mRNA transcription and steady state levels. This inhibition of transcription occurs without abrogating the binding of the 1α,25-$(OH)_2D_3$ receptor complex to the vitamin D-responsive element (VDRE) of the OC gene. Since 1β,25-$(OH)_2D_3$ has no genomic effects on OC but can block the rapid nongenomic effects of 1α,25-$(OH)_2D_3$ and accompanying increase in OC mRNA transcription, the data suggest that the rapid nongenomic effects of 1α,25-$(OH)_2D_3$ may modulate the genomic actions of the hormone on OC mRNA transcription.

Received May 4, 1992; accepted May 28, 1992.

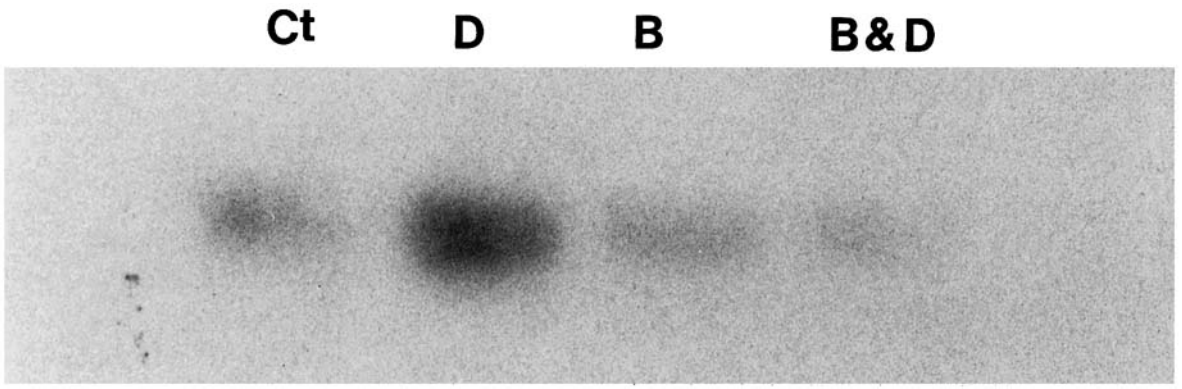

Fig. 1. Northern analysis of osteocalcin mRNA. Total cellular RNA was isolated from ROS 17/2.8 cells exposed to vehicle (Ct), 1α,25-$(OH)_2D_3$ 20 nM (D), 1β,25-$(OH)_2D_3$ 20 nM (B), or both (B & D) and hybridized to rat osteocalcin genomic clone, pOC 3.4. Uniformity of RNA application was confirmed by quantification of ribosomal RNA hybridized with ^{32}P-labeled 28 s ribosomal DNA genomic clone, LS-6.

METHODS

Materials

Osteoblast-like rat osteosarcoma cells, ROS 17/2.8 (generously supplied by Dr. G. Rodan, Merck Sharp Dohme, West Point, PA), were cultured 6–7 days and harvested as previously described [Baran et al., 1991]. The 1α,25-$(OH)_2D_3$ was provided courtesy of Dr. M. Uskovic [Hoffmann–LaRoche Inc., Nutley, NJ], while 1β,25-$(OH)_2D_3$ was provided courtesy of Dr. M. Holick of Boston University (Boston, MA).

Assays

1. Determination of intracellular Ca^{2+} levels by Quin 2 fluorescence was measured as previously described [Baran et al., 1991]. The ROS cells, 1×10^8, were incubated for 1 hr in the presence and absence of 1α,25-$(OH)_2D_3$, 20 nM, or its inactive epimer, 1β,25-$(OH)_2D_3$, 20 nM. Cells were loaded with Quin 2AM (CalBiochem-Behring Corp., San Diego, CA), 1,000 nmoles, in 10 μl of dimethylsulfoxide (DMSO), for the last 30 min of the incubation. Cells were washed and treated with 1α,25-$(OH)_2D_3$ or its inactive epimer or the two in combination and fluorescence was measured. In those experiments in which intracellular Ca^{2+} was measured after 3-hr exposure to 1α,25-$(OH)_2D_3$, the quin 2AM was added as above but after 2½-hr treatment with the hormone.

2. Steady-state mRNA levels of OC were measured in total cellular RNA isolated from ROS 17/2.8 cells exposed to 1α,25-$(OH)_2D_3$, 20 nM, or 1β,25-$(OH)_2D_3$ for 3 hr. RNA was extracted by the commercial RNAzol method (Cinna/Biotecx Laboratories, Friendswood, TX). OC mRNA levels were analyzed by Northern blot following hybridization to rat OC genomic clone, pOC 3.4 [Lian et al., 1989].

3. Transcription was assayed in isolated nuclei in the presence of ^{32}P-labeled UTP as reported [Greenberg and Ziff, 1984]. Radiolabeled RNA transcripts were hybridized to slot blots of cloned pOC 3.4 and ribosomal genes.

4. Gel mobility shift assays were carried out with nuclear proteins prepared from ROS 17/2.8 cells as described [Dignam et al., 1983; Holthuis et al., 1990] pretreated for 24 hr with 1α,25-$(OH)_2D_3$ and/or 1β,25-$(OH)_2D_3$. ^{32}P-labeled VDRE probe (an oligonucleotide spanning nt-466 to -437 of the rat OC promoter) was prepared by labeling 100 ng of one strand of the oligonucleotide using ^{32}P-ATP and polynucleotide kinase, annealing 200 ng of the complementary unlabeled strand and purifying the double-stranded oligonucleotide by polyacrylamide gel electrophoresis (PAGE).

Statistics

Values represent the mean ± SD, and probability of difference was determined by Student's *t*-test or Duncan's test for multiple comparisons where indicated.

RESULTS

Treatment of ROS 17/2.8 cells for 3 hr with 1α,25-$(OH)_2D_3$ significantly increases intracellular Ca^{2+} (32 ± 5 vs. 58 ± 8 nM, $P < 0.05$). Although 1β,25-$(OH)_2D_3$, 20 nM, has no effect on intracellular Ca^{2+} (32 ± 9 nM) it inhibits the 1α,25-$(OH)_2D_3$-induced increment (37 ± 9 nM).

TABLE I. Osteocalcin mRNA Levels in ROS 17/2.8 Cells Treated With 1α,25-$(OH)_2D_3$, 1β,25-$(OH)_2D_3$, or the Combination for 3 hr†

Control	1α,25-$(OH)_2D_3$ 20 nM	1β,25-$(OH)_2D_3$ 20 nM	1α,25-$(OH)_2D_3$ 20 nM + 1β,25-$(OH)_2D_3$ 20 nM
0.96 ± 0.05	1.61* ± 0.22	0.82 ± 0.30	1.02 ± 0.36

†Values represent the mean ± SD of three observations in each group and are expressed as a function of the ribosomal RNA content.
*$P < 0.05$ compared to other groups by Duncan's test for multiple comparisons.

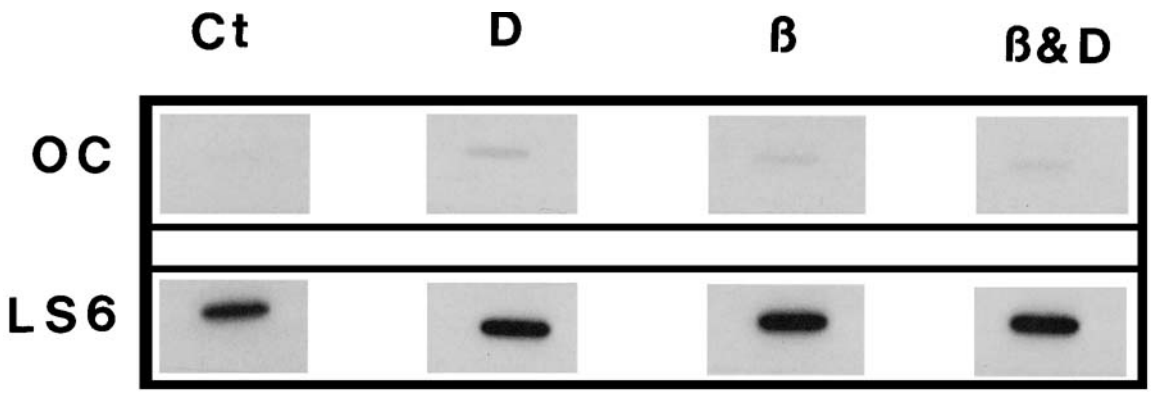

Fig. 2. Nuclear run-on assay of osteocalcin mRNA transcription. ROS 17/2.8 cells were treated with vehicle (Ct), 1α,25-$(OH)_2D_3$, 20 nM (D), 1β,25-$(OH)_2D_3$, 20 nM (B), or both (B&D) for 1 hr. Isolated nuclei were transcribed in the presence of ^{32}P-UTP and radiolabeled RNA transcripts hybridized to slot blots of cloned pOC 3.4 and ribosomal genomic clone LS-6.

1α,25-$(OH)_2D_3$, 20 nM, increases OC mRNA levels in ROS 17/2.8 cells after 3 hr by 67% (Fig. 1, Table I). 1β,25-$(OH)_2D_3$, 20 nM, has no effect on OC mRNA levels. However, just as 1β,25-$(OH)_2D_3$ blocks the 1α,25-$(OH)_2D_3$-induced increase in intracellular calcium, it also inhibits the 1α,25-$(OH)_2D_3$-induced increment in OC gene expression (Fig. 1, Table I).

Treatment of ROS 17/2.8 cells for 1 hr with 1α,25-$(OH)_2D_3$, 20 nM, increases intracellular Ca^{2+} (40 ± 5 vs. 60 ± 13 nM, $P < 0.05$). 1β,25-$(OH)_2D_3$, 20 nM, has no effect on intracellular Ca^{2+} (37 ± 7 nM) after 1 hr but inhibits the hormone-induced increment (37 ± 6 nM). 1α,25-$(OH)_2D_3$ increases OC mRNA transcription after 1 hr (Figs. 2, 3). Similar to its effect on cellular Ca^{2+}, and OC mRNA steady-state levels 1β,25-$(OH)_2D_3$ also blocks the 1α,25-$(OH)_2D_3$-induced increase in OC mRNA transcription (Figs. 2, 3).

To determine if the 1α,25-$(OH)_2D_3$-induced increments in cell Ca^{2+} were affecting the sequence specific binding of the vitamin D-receptor complex to the VDRE of the OC gene, cells were incubated in the presence of 1α,25-$(OH)_2D_3$, 1β,25-$(OH)_2D_3$, or the combination for 24 hr. As shown in Figure 4, nuclear extracts from ROS 17/2.8 cells treated with 1α,25-$(OH)_2D_3$ reveal increased sequence specific binding to the VDRE (Fig. 4D). Inhibiting the nongenomic effects of 1α,25-$(OH)_2D_3$ with the 1β-epimer did not prevent the sequence specific binding of the hormone receptor complex (Fig. 4BD).

DISCUSSION

1α,25-$(OH)_2D_3$ increases OC message and OC gene transcription [Lian et al., 1989; Pan and Price, 1984; Markose et al., 1990, Demay et al., 1989; Kemer et al., 1989]. This study shows that hormone induced rapid, nongenomic actions accompany and may be functionally related to the increases in OC mRNA transcription and steady-state levels. We have shown that 1β,25-$(OH)_2D_3$ blocks the 1α,25-$(OH)_2D_3$-induced increase in intracellular Ca^{2+} and OC mRNA transcription. 1β,25-$(OH)_2D_3$ does not bind to nor displace 1α,25-$(OH)_2D_3$ from its nuclear receptor [Holick et al., 1980] but does compete with 1α,25-$(OH)_2D_3$ for the rapid signaling system regulating the nongenomic effects [Baran et al., 1991]. The data in this paper indicate that the rapid actions may have functional significance in gene transcription. The 1β-epimer does not appear to affect the binding of the vitamin D-receptor

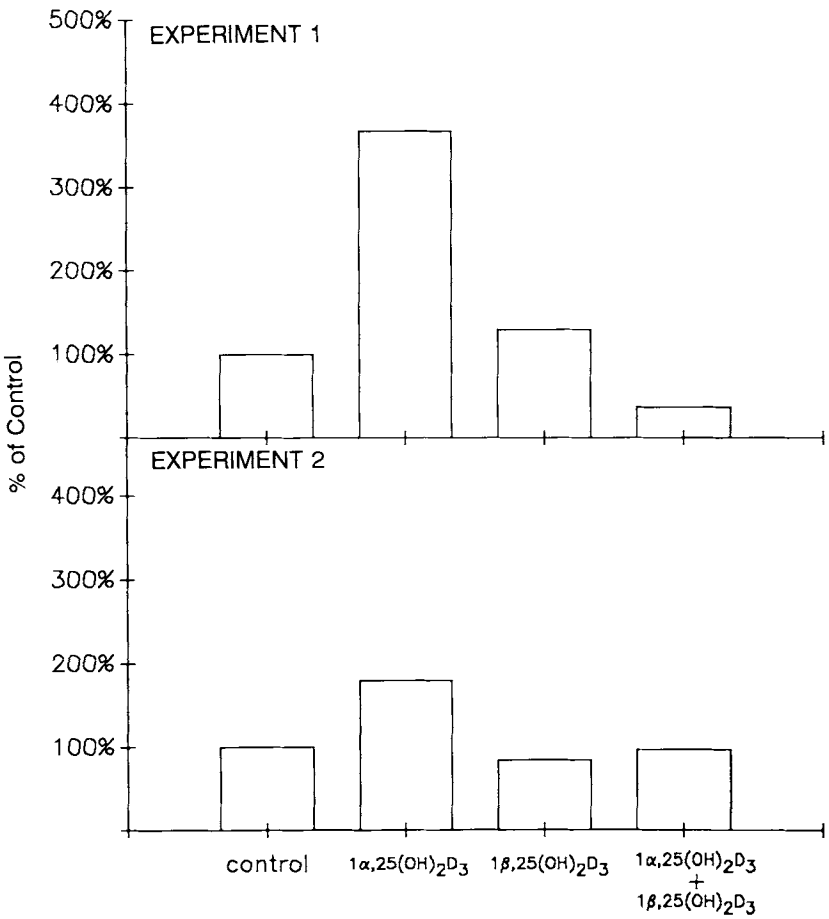

Fig. 3. Osteocalcin mRNA transcription. ROS 17/2.8 cells were treated and the nuclei transcribed. These changes were reproducible in independent experiments.

complex to the VDRE of the OC gene (Fig. 4); however, the manner in which the complex interacts with its cognate binding sequence and/or other proteins associated with the VDRE region may be altered. The mechanism(s) by which the rapid effects modulate these genomic actions of $1\alpha,25\text{-}(OH)_2D_3$ is unclear.

Structure function studies using ligand analogs of $1\alpha,25\text{-}(OH)_2D_3$ suggest that there may exist distinct nuclear receptors and plasma membrane-associated forms of the $1\alpha,25\text{-}(OH)_2D_3$ receptor that are involved in genomic and nongenomic activation of osteoblastic activity [Farach-Carson et al., 1991]. Thus, a signalling system separate from the classical vitamin D-receptor appears to be involved in the rapid effects of $1\alpha,25\text{-}(OH)_2D_3$ in rat osteosarcoma cells. Recent studies have demonstrated that phosphorylation of the vitamin D nuclear receptor is a necessary event for hormone action [Brown and Deluca, 1990]. Phosphorylation of the receptor on serine 51 by protein kinase C is not necessary for binding of the hormone receptor complex to the VDRE but is crucial to its transactivation function [Hsieh et al., 1991]. Therefore, if the rapid nongenomic effects of $1\alpha,25\text{-}(OH)_2D_3$ are necessary for protein kinase C-induced phosphorylation of the vitamin D receptor, inhibition of these increments by $1\beta,25\text{-}(OH)_2D_3$ could affect transcription of OC mRNA without altering binding of the hormone receptor complex to the VDRE. Another possible consideration is that transcription factor binding to an element other than the VDRE that influences OC gene transcription may be affected.

An alternative mechanism may involve the rapid effects of $1\alpha,25\text{-}(OH)_2D_3$ on phospholipid metabolism in plasma and nuclear membranes [Civitelli et al., 1990; Baran et al., 1989], which might contribute to the hormone's transactivation function. Inositol trisphosphate, produced in response to $1\alpha,25\text{-}(OH)_2D_3$ in rat osteosarcoma cells [Civitelli et al., 1990], stimulates protein kinase C-dependent protein phosphory-

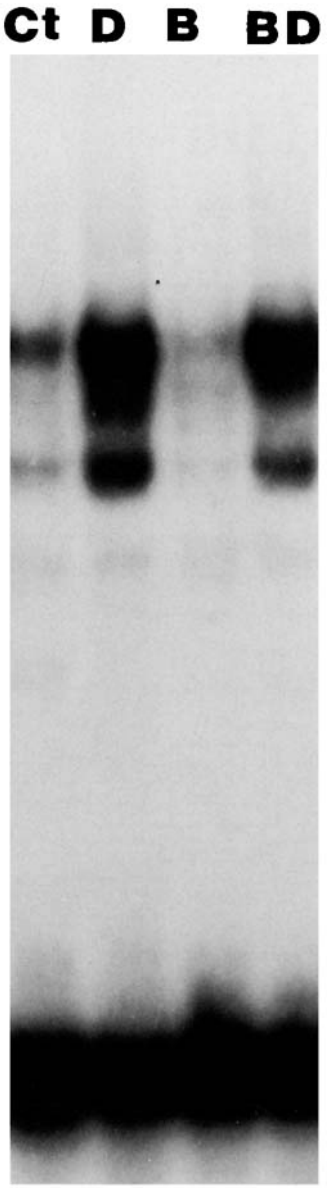

Fig. 4. Gel mobility shift assay of nuclear extracts from ROS 17/2.8 cells. Cells were treated for 24 hr with vehicle (Ct), 1α,25-$(OH)_2D_3$, 20 nM (D), 1β,25-$(OH)_2D_3$ 20 nM (B), or both (BD). Sequence specific binding to the VDRE of the osteocalcin gene is increased by treatment with 1α,25-$(OH)_2D_3$ **(lane B)**, and unaffected by 1β,25-$(OH)_2D_3$ **(lane Ct)**. 1β,25-$(OH)_2D_3$ does not affect the 1α,25-$(OH)_2D_3$-induced increase in binding **(lane D)**.

lation in isolated nuclei [Martelli et al., 1990]. Nuclear phospholipids appear to be responsible for the hydrophobic interactions between nucleic acids and nuclear matrix fibrils [Cocco et al., 1980]. 1β,25-$(OH)_2D_3$ inhibits the rapid effects of 1α,25-$(OH)_2D_3$ on phospholipid metabolism in hepatocytes [Baran et al., 1990] perhaps explaining the ability of 1β,25-$(OH)_2D_3$ to inhibit OC gene transcription induced by 1α,25-$(OH)_2D_3$. Lastly, the increase in transcriptional responsiveness to Ca^{2+} is consistent with a 100-fold up-regulation of OC gene expression in mineralizing chick and rat osteoblast cultures [Aronow et al., 1990]. Extracellular Ca^{2+} and 1α,25-$(OH)_2D_3$ acting together stimulate OC release by primary human bone cells [Lajeunesse et al., 1991] and modulate the expression of the calbindin gene in kidney [Clemens et al., 1989] and intestine [Brehier et al., 1989].

In ROS cells, 1α,25-$(OH)_2D_3$ also exerts nongenomic effects on intracellular Ca^{2+} and phospholipid metabolism through a signalling system that differs from the classic vitamin D receptor [Civitelli et al., 1990; Baran et al., 1991; Farach-Carson et al., 1991]. The results of this study demonstrate that one genomic effect of 1α,25-$(OH)_2D_3$ in ROS 17/2.8 cells, increased OC mRNA transcription, is potentially modulated by these rapid effects. Thus, this rapid, nongenomic, separate signaling system appears to be functionally involved in the genomic effects of 1α,25-$(OH)_2D_3$ in ROS 17/2.8 cells by a yet to be defined mechanism.

ACKNOWLEDGMENTS

This work was supported in part by grants DK39085, AR39588, and AR33920 from the NIH. An abstract of this work was presented at the American Society for Bone and Mineral Research, San Diego, 1991.

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